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Physiological Aspects of Free-Radical Reactions

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Enzymes which catalyze the formation of free radicals *in vitro* will catalyze similar reactions *in vivo*. We believe that the formation of some kinds of free radicals has definite physiological meanings in metabolism. In this sense, the enzymes forming such free radicals are concluded to be in evolutionally advanced states. Elaborated structure and function of enzymes such as horseradish peroxidase and microsomal flavoproteins support the idea. Deleterious and side reactions caused by free radicals are assumed to be minimized *in vivo* by localizing the reactions, but this assumption should be verified by future studies.

Free radicals are regarded to be toxic to cells and have been studied mostly to elucidate their deleterious effects in cells. This tendency of studies of free radicals has been strengthened since the finding of McCord and Fridovich (*1*), who reported an enzyme that decomposes superoxide radicals. Numerous experimental results have led one to conclude that the enzyme called superoxide dismutase is an indispensable component of the system of defenses which make aerobic life possible (*2–5*). Semidehydroascorbate reductase is another type of free-radical scavenging enzyme, which has been studied a long time (*6–13*). In this case, however, it is not known whether this enzyme also serves as a defense enzyme.

An enzyme is believed to catalyze a unique reaction which has a definite physiological role or an evolutional meaning in biology. For instance, the relationship between structure and function of oxygen-metabolizing enzymes has been explained in terms of evolution of enzymes (*14*). There are a group of enzymes which catalyze the formation of free radicals. It seems, therefore, of primary importance to discuss the question whether enzymatic formation of free radicals has any physiological meanings. If such free radicals are really deleterious, the free-radical formation may be a defect in the enzyme catalysis, which is still in an evolutionary stage. We shall treat the problem from the two points of view; mechanism of enzymatic formation of free radicals and possible physiological meanings of free-radical reactions.

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Mechanism of Free-Radical Formation

In some enzymatic redox reactions, free radicals are derived from substrates and prosthetic groups of enzymes. Enzymes which catalyze free-radical reactions contain transition metal ion(s), flavin(s) or rarely quiones(s) as the prosthetic group. These enzymes are grouped into apparently two contrasting types, one that scavenges free radicals using them as a substrate and the other that catalyzes the formation of free radicals from a substrate. Superoxide radical and semidehydroascorbate are the only known free radicals used as the substrate of enzymes, while many free radical species are reported to be formed during enzyme catalysis (Table 1). Free radicals derived from substrates usually decay fast through a dimerization or dismutation pathway and accumulate only a little during the reaction. It is not always possible to detect them directly with an optical or ESR spectrometer. In order to analyze the mechanism of electron transfer between enzyme and substrate, it is necessary to clarify whether free radicals are primary products in enzyme catalysis and whether they are formed through a main enzyme reaction. The experimental methods for the quantitative measurement of one-electron flux in enzymatic redox reactions were discussed in detail previously (*41,42*). After careful consideration of the above problems and on the basis of quantitative analysis, it is possible to classify the electron-transfer mechanism into one-electron and two-electron types. In a few cases, the electron transfer occurs through a mixed mechanism (*41,42*).

Then, the question may arise whether any unique structural factors are needed for enzymes to catalyze the formation of substrate free radicals. Since it is known that the superoxide radical is formed through autoxidation of various divalent reductants, nonenzymatic electron transfer might be by nature a one-electron type. Therefore, it may also be necessary to consider the evolutional state of free-radical-forming enzymes from the points of catalytic efficiency and specificity.

Here, we shall discuss kinetics of enzymatic and nonenzymatic electron transfers, and enzymatic devices which may control the formation of free radicals.

Nonenzymatic Formation of Free Radicals

There are numerous reports concerning the formation of free radicals in nonenzymatic reactions (*43*). It is beyond doubt that a free radical is formed when a divalent molecule is oxidized or reduced by a monovalent molecule. The problem to be discussed here is how much free radicals are formed in electron-transfer reactions between two divalent molecules. For example, the primary process of autoxidation of a divalent reductant (AH_2) will be formulated as shown in Eqs. (1)-(3).

$$AH_2 + O_2 \rightarrow AH\cdot + HO_2\cdot \quad (1)$$

$$AH_2 + O_2 \rightarrow A + H_2O_2 \quad (2)$$

$$AH_2 + O_2 \rightarrow x(AH\cdot + HO_2\cdot) + y(A + H_2O_2) \quad (3)$$

where $x + y = 1$. The mechanism of the reactions is termed, from the top, one-electron, two-electron and mixed types (*41*). The fraction of one-electron flux, $x/(x+2y)$ has to be measured for characterization of reaction (3) (*42*). The formation of superoxide radical has been shown in autoxidations of reduced flavin (*44,45*), pyrogallol (*46*), dyes (*47,48*), adrenalin (*49,50*), and ascorbate (*51*). The amount of superoxide radical formed during the reaction is measured directly with an ESR spectrometer (*44*) or indirectly from secondary reactions of the superoxide radical with electron acceptors and adrenalin (*45–50*). The difficulty of quantitative measurement of one-electron flux is due to nonspecific reactions caused by free radicals.

A kinetic study has been carried out on a reaction between ascorbate and 2,6-dichlorophenolindophenol (DCIP). During the oxidation of ascorbate by DCIP, only ascorbate free radical is observed. The dependency of steady-state concentration of ascorbate free radical on the DCIP concentration is shown in Figure 1 (*52*). Based on the rate constant measured for dismutation of ascorbate free radicals (*15,53–55*) it can be concluded that ascorbate is oxidized mostly by way of one-electron transfer, the one-electron flux being about 80%. Figure 1 shows also that ascorbate free radicals decay mostly by dismutation at low concentrations of DCIP and the decay through a reaction with DCIP increases as the dye concentration increases.

$$AH_2 + DCIP \rightarrow AH\cdot + DCIPH\cdot \quad (4)$$

$$2AH\cdot \rightarrow A + AH_2 \quad (5)$$

$$2\ DCIPH\cdot \rightarrow DCIP + DCIPH_2\ \text{(leuco-form)} \quad (6)$$

$$AH\cdot + DCIP \rightarrow A + DCIPH\cdot \quad (7)$$

where AH_2 denotes ascorbic acid. The DCIP free radical, DCIPH·, appears to decay very fast because the ESR signal observed is only that of ascorbate free rad-

Table 1. Free radicals generated from substrates by enzymes.

Free radical	Enzyme		Reference
	Oxidative	Reductive	
Semidehydroascorbate	Peroxidase		(*15-17*)
	Ascorbate oxidase		(*15*)
	Dopamine β-monooxygenase		(*18*)
Semidehydroreductate	Ascorbate oxidase		(*15*)
Semiquinones	Laccase		(*19*)
	Peroxidase		(*17,20,21*)
		Microsomal flavorproteins	(*8*)
		NADH dehydrogenase	(*22*)
		Ferredoxin-NADP reductase	(*22*)
		Xanthine oxidase	(*23*)
		Xanthine dehydrogenase	(*24*)
		Lipoamide dehydrogenase	(*25*)
Superoxide anion		Xanthine oxidase	(*26,27*)
		Flavoproteins in phagocytosis	
Phenoxy radicals	Peroxidase		(*17,28–30*)
Free radicals from			
Amines	Peroxidase		(*31–33*)
	Prostaglandin endoperoxide synthetase		(*34–37*)
	Cytochrome P-450		(*38*)
Chlorpromazine	Peroxidase		(*39*)
Indoleacetate	Peroxidase		(*40*)

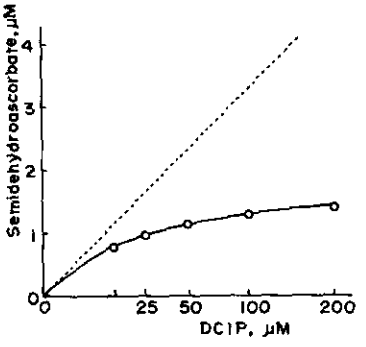

FIGURE 1. Steady-state concentration of semidehydroascorbate formed in the oxidation of ascorbate by 2,6-dichlorophenolindophenol (DCIP) at varied concentrations: (--) calculated on the assumption that ascorbate is oxidized to semidehydroascorbate, which decays only through dismutation.

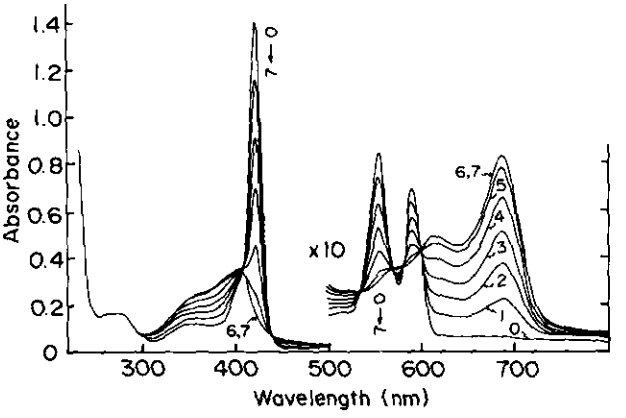

FIGURE 3. Oxidation of Mg-substituted horseradish peroxidase by potassium hexachloroiridiate and formation of porphyrin π-cation radical. The Mg enzyme (0) is titrated stepwise with hexachloroiridate, and the final spectrum (*6,7*) is obtained at the equimolar concentration (*78*).

ical. Although Misra and Fridovich (*45*) have reported two-electron reduction of oxygen by reduced menadione, it may be concluded that free-radical species are more or less formed in nonenzymatic electron transfers between two divalent redox molecules.

Enzymatic Formation of Free Radicals

For most enzymatic reactions it is possible to estimate the exact fraction of one-electron flux (*41,42*). The mechanism of electron transfer can be concluded to depend mostly on the structure of enzymes, including interaction between protein and prosthetic groups. For instance, ascorbate oxidase, laccase and tyrosinase are all copper proteins, but the former two catalyze one-electron oxidations of donor molecules (*15,19*) and tyrosinase catalyzes a two-electron oxidation of catechol (*56*). The oxygen is freed from these copper enzymes after it is reduced to water. It is, therefore, concluded that the electron-transfer mechanism is not determined by prosthetic group itself. This conclusion is valid for heme- and flavin-containing enzymes.

The most characteristic feature of enzyme catalysis which yields free radicals may be that free radicals are derived from only one of the two substrates, oxidant and reductant, but not from both of them (*42*). It is also important to note that an electron-transfer mechanism

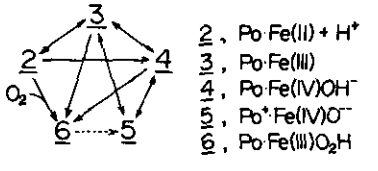

FIGURE 2. Five oxidation states of horseradish peroxidase: (2) ferrous; (3) ferric; (4) Compound II or ferryl; (5) compound I or ferryl + porphyrin π-cation radical; and (6) compound III or oxyform. Po denotes porphyrin. Correlation is shown in Table 2.

is not necessarily fixed to an enzyme. In some cases, the mechanism varies with the kind of substrate and the experimental conditions (*41,42*).

Horseradish Peroxidase. Peroxidases distributed widely in tissues of plants are similar to each other and can be represented by horseradish peroxidase, which has been studied extensively (*57,58*). The enzyme has five oxidation states from 2 to 6, where the oxidation state of ferrous enzyme is set at 2 (*74*). The conversion between these states is caused by suitable oxidants and reductants, as shown in Figure 2 and Table 2. The reactions are essentially the same as those of animal peroxidases prepared from thyroid, milk, intestine and leucocyte. The 5 state of animal peroxidases, however, is unstable and converted to 4 within a second, probably by endogenous reductants. Characteristic of horseradish peroxidase regarding the formation of free radicals is as follows.

In the 3 → 5 reaction, upon the reaction of the 3 state with hydroperoxides (ROOH), the O—O bond of ROOH is split heterolytically by acid-base catalysis of the iron and two amino acid residues in the heme crevice (*75*). The two oxidizing equivalents of ROOH are retained at the heme moiety, one as a ferryl ion and the other as a porphyrin π-cation radical (*76*). This radical can be detected quantitatively by optical and ESR spectroscopy when the iron of horseradish peroxidase is replaced by Zn or Mg (*77,78*). Spectrophotometric titration with potassium hexachloroiridate is shown in Figure 3. As the porphyrin π-cation radical is not formed in myoglobin, it is concluded that the heme crevice of peroxidase has a structure suitable for stabilizing the porphyrin π-cation radical (*77,78*).

In the 5→4→3 and 5→3 reactions, various electron donors are oxidized by 5, via a route of either 5→4→3 or 5→3. The mechanism is dependent on the kind of electron donors and, in a few cases, on pH also. The 5→4→3 mechanism is common to most peroxidase re-

Table 2. Correlation between five oxidation states of peroxidase.

Reaction[a]	Oxidant	Reductant	Reference
2→3	Ferricyanide		(*59*)
2→4	H_2O_2		(*60*)
2→6	O_2		(*61,62*)
3→2		Dithionite	(*59*)
3→4	Hexachloroiridate, *ferricyanide*[b]		(*63*)
3→5	H_2O_2, alkylhydroperoxides, peracids		(*59,64,65*)
3→6	Superoxide		(*23,66,67*)
4→3		Numerous donors	[c]
4→5	Hexachloroiridate		(*63*)
4→6	H_2O_2		(*68*)
5→3		Iodide, thiocyanate H_2O_2, sulfite [d]	(*69–71*)
5→4	Numerous donors		[c]
6→5		Hydrated electron	(*72*)
6→3[e]		*p*-Phenylenediamine	(*73*)

[a] Numbers denote oxidation states (See Fig. 2).
[b] Only at alkaline pH.
[c] See Table 1.
[d] At acidic pH.
[e] The reaction appears to occur via 6→5→4→3, although the intermediates are not detected.

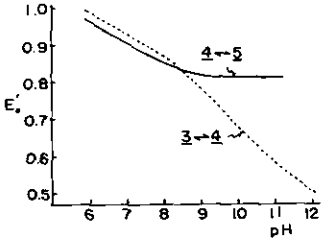

FIGURE 4. pH dependence of redox potentials of horseradish peroxidase (*63*).

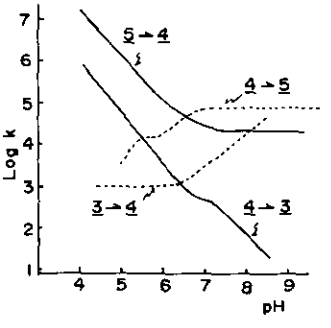

FIGURE 5. Second-order rate constants *k* for oxidation by potassium hexachloroiridate and reduction by potassium hexachloroiridite of horseradish peroxidase A_2 (*63*).

actions and electron donors are all oxidized to free radicals. For the same electron donor, the 5→4 reaction is approximately 100 times faster than the 4→3 reaction (*79,80*). This difference cannot be explained in terms of redox potentials because the E'_0 values for the 5/4 and the 4/3 couples are about the same at pH values where the enzyme activity is measured (Fig. 4). The relation between the rate constant and the redox potential is demonstrated in the result of Figure 5, which shows that the oxidation of 4 by hexachloroiridate is faster than that of 3 by hexachloroiridate (*63*). These results have led us to conclude that an electron is transferable from and to porphyrin much faster than from and to the heme iron. One of the reasons may be that the edge of porphyrin ring in peroxidases is exposed to the solvent. And, this might be a structural factor that enables peroxidases to catalyze typical one-electron oxidations of donor molecules. In most cases, peroxidases exist as the 4 form during the catalytic reactions because the 3→5 reaction is very fast under ordinary experimental conditions. Free radicals formed during the reactions have been measured by ESR spectroscopy (*15–17,20,21,28,29,31,33,39,81,82*).

The oxidations of halides, thiocyanate and hydrogen peroxide are coupled with the 5→3 path of peroxidases, occurring by way of two-electron transfer (Table 2). Horseradish peroxidase catalyzes the oxidation of sulfite through a mixed mechanism, which varies from a two-electron type at acidic pH to a one-electron type at alkaline pH (*69*). Although it is in general true that peroxidases catalyze one-electron oxidations of organic molecules, such as phenol derivatives and aromatic amines, an interesting exception has been found in the reactions of thyroxine synthesis catalyzed by thyroid peroxidase, which will be discussed later.

The 3→6 reaction was suggested to explain the stoichiometric formation of 6 during the aerobic oxidation of dihydroxyfumarate catalyzed by horseradish peroxidase (*66*). The formation of 6 from 3 + O_2^{-}• has been confirmed with lactoperoxidase (*23*), intestinal tryptophan 2,3-dioxygenase (*83*) and myeloperoxidase (*67*), and recently by pulse radiolysis with horseradish peroxidase (*84*) and catalase (*85*). Since the reaction occurs with a rate constant of 10^6–10^7/M-sec (*86,87*), it can be used for the measurement of the amount of superoxide radical formed (*23*). As the stability of 6 is dependent

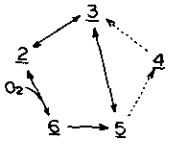

FIGURE 6. Pentagonal representation of the function of cytochrome P-450 in comparison with that of horseradish peroxidase.

on the experimental conditions, this method is not valid for some cases (*88*).

Cytochrome P-450. Cytochrome P-450 is a unique monooxygenase. Since it has a peroxidase activity (*89*) and a spectral property similar to chloroperoxidase (*90*), the structural and functional comparison of cytochrome P-450 with peroxidases is an interesting subject (*91–93*). The catalytic pathway of cytochrome P-450 can also be shown in a pentagonal diagram (Fig. 6). As compared with peroxidases, several important points still remain to be solved in the case of cytochrome P-450 (Table 3). Porphyrin π-cation radical is formed in peroxidases, but is not detected in cytochrome P-450. The ferryl form is a common catalytic intermediate of peroxidases, and a similar form is found in cytochrome P-450 (*95*) but its participation is uncertain in cytochrome P-450 reactions. The cleavage of the O—O bond accompanying the reactions of 6→5 and 3 + ROOH is heterolytic in peroxidases, but is still a controversial subject in cytochrome P-450 (*96–98*).

The formation of aminopyrine free radical is directly confirmed by optical and ESR spectroscopic methods in cytochrome P-450 reactions (*99,100*). The involvement of free radical intermediate is also confirmed with the uses of spin traps (*101–103*) and radical scavengers (*103–105*). Particularly, several workers have concluded that the hydroxyl radical which is formed through an iron-catalyzed Haber-Weiss reaction causes the hydroxylation of aniline (*106*) and benzene (*107*), and the oxidation of ethanol (*108–111*).

$$O_2^{-}\cdot + H_2O_2 \xrightarrow{\text{(iron complex)}} O_2 + HO^{-} + HO\cdot \qquad (8)$$

The source of superoxide radical may be either the autoxidation of cytochrome P-450 reductase (*110*) or of cytochrome P-450 (*106,107,111,112*).

The key problem in the mechanism of cytochrome P-450 reactions is whether cleavage of the O—O bond is heterolytic or homolytic. As the homolytic cleavage yields the hydroxyl radical, it is inconsistent with the mechanism involving the Haber-Weiss pathway in the formation of hydroxyl radical. On the other hand, the heterolytic cleavage is accepted by workers who insist an oxene-transfer mechanism for hydroxylation by cytochrome P-450 (*113,114*). In the heterolytic cleavage, like peroxidases, cytochrome P-450 is to be oxidized to the 5 form (compound I) upon reactions of 6→5 and 3 + ROOH. Then, in cytochrome P-450 reactions, the oxygen atom coordinated at the 6th position of heme iron is to be transfered to an oxygen acceptor. Although the formation of a higher valence state of cytochrome P-450 is reported (*95,114*), there is no direct evidence to support the oxene-transfer mechanism.

Another serious question as to the mechanism of cytochrome P-450 reactions would be the time of the OO bond cleavage. In the oxene mechanism, the heterolytic cleavage is to precede the oxygen transfer. In the mechanism of homolytic cleavage, the 5 form is regarded as a complex of 3 with hydroperoxide (*96,97*) and the O—O bond cleavage will be accompanied with the hydroxylation. A free radical derived from the homolytic cleavage of the O—O bond abstracts a hydrogen atom from an oxygen acceptor (RH) and the R$^{\cdot}$ thus formed reacts with the iron-bound hydroxyl radical to produce ROH. After a series of these reactions in the heme crevice a hydroxylated molecule will be released from the enzyme. This mechanism, which may be called an intramolecular free-radical mechanism, will explain various aspects of liver microsomal cytochrome P-450 reactions (*97,98,115*). It seems possible that electron donors and spin-trapping reagents are released from the enzyme as free radicals just after reacting with free-radical species formed by the homolytic cleavage. The flexible mechanism of liver microsomal cytochrome P-450 will be related to its low specificity for substrate. Since the release of free radicals does not appear to be physiological in this case, such flexibility of the mechanism is of special interest from the physiological and evolutional points of view.

Flavoprotein Enzymes. Flavoproteins occupy key positions in biological electron transport systems. In general, a flavoprotein is reduced by a specific substrate

Table 3. Comparison between peroxidase and cytochrome P-450.

	Peroxidase	Cytochrome P-450
Heme	Protoheme[a]	Protoheme
5th ligand	Imidazol[b]	Thiol
Cleavage of the O-O bond	Heterolytic	Homolytic and coupled with hydroxylation[c]
5 state	Ferryl plus porphyrin π-cation radical[d]	complex of 3 with hydroperoxide?
4 state	Ferryl	Not confirmed[e]

[a] A slightly modified form of protoheme is involved in animal peroxidases except for myeloperoxidase which contains a formyl heme.
[b] The 5th ligand of chloroperoxidase is thiol.
[c] See McCarthy and White (*93*)
[d] The oxidizing equivalent is transferred from the porphyrin to an amino acid residue in the case of cytochrome c peroxidase (*94*).
[e] See text.

and reoxidized by relatively nonspecific electron acceptors although *in vivo* it mediates a specified electron transfer from donor to acceptor. The mechanism of electron transfer from donor to flavoprotein is regarded to be a hydride or two-electron transfer (*41,116*) and the one-electron transfer mechanism is frequently involved in the reactions between flavoproteins and electron acceptors. Of numerous electron acceptors, *p*-benzoquinone serves as good electron acceptor for most flavoproteins, and the fraction of one-electron reduction can easily be measured by either ESR or scavenger method (*8,22,41,42*).

The dependence of the one-electron flux on the experimental conditions is summarized in Table 4. The results suggest that (i) when the one-electron flux depends on pH, it increases as the pH increases; (ii) when the one-electron flux depends on the acceptor concentration, it increases as the acceptor concentration increases; (iii) the one-electron flux increases when the enzyme suffers mild modification. Although it is premature to deduce generalization from a few examples, there are reasons to rationalize the rules. For (i), upon the stepwise reduction of oxygen or quinone (A) at neutral pH, the two protons are incorporated at the second step.

$$A \xrightarrow{e^-} A^{-}\cdot \xrightarrow{e^- + 2H^+} AH_2 \qquad (9)$$

The release of AH_2 becomes difficult as the pH increases. For (ii), if the rate of electron supply to a flavoprotein is limited, the one-electron transfer from the flavoprotein to A will predominate over the two-electron transfer as the concentration of A increases. This can be seen in the reaction of xanthine oxidase in which molybdenum and iron ions are contained in addition to flavin. For (iii), the two-electron transfer tends to occur in a reaction of an enzyme with a highly specific substrate and the loosening its interaction will result in a shift from two-electron to one-electron transfer mechanism.

We have shown that the primary reduction products of quinones are semiquinones in the reactions catalyzed by flavoprotein enzymes of electron transport systems and hydroquinone forms in the reaction catalyzed by DT diaphorase (*8,22*). The difference is not completely explained in terms of molecular structure of the flavoproteins, but clear experimental evidence has been given to answer the question why microsomal flavoproteins catalyze one-electron reduction of quinones (*119–121*). Cytochrome P-450 reductase contains two flavins, FAD and FMN (*119*). The FMN acts like flavodoxin and is converted to a stable FMNH• form during the reaction (*119,120*). Cytochrome b_5 reductase contains only FAD, but the FADH• form is stabilized in the presence of pyridine nucleotide (*121*). Therefore, in the NADH-quinone reductase reaction the reduced enzyme ($FADH_2$) is reoxidized by a quinone via FADH• and the semiquinone is released. Of course, *in vivo*, the electron acceptor is cytochrome in either case, and the reaction with quinones is artificial. At any rate, it is likely that the formation of stable flavin semiquinone is prerequisite to the one-electron transfer mechanism.

Possible Roles of Free-Radical Reactions

A molecule in biological systems is activated and undergoes an ordered reaction at an active site of an enzyme. Active sites of oxido-reductive enzymes consist of prosthetic groups and functional aminoacid residues. There are two major groups of enzymes which catalyze the formation of free radicals. One is flavin-containing enzymes present in electron transport systems and the other is heme-containing enzymes, peroxidases, and peroxidase-like enzymes. The physiological meanings of free radical formation appear to be quite different between these two groups of enzymes.

Energy Conservation

Flavoproteins present in electron transport systems catalyze one-electron transfer to cytochromes or quinones. The one-electron flux is 100% even when qui-

Table 4. Factors which increase the one-electron flux in some enzyme catalyses.

Enzyme	Reactions[a]	Condition	Reference
Xanthine oxidase	Xanthine–O_2	$\Delta[O_2]$ (5 to 50%)[b]	(*24,27*)
		ΔpH (15 to 7%)	(*24,27*)
	Xanthine–Q	Δ[Q](0 to 100%)	(*23,117*)
		Deflavo (100%)	(*117*)
		Dehydrogenase type (100%)[c]	(*118*)
Lipamide dehydrogenase	NADH–Q	ΔpH (15 to 80%)[d]	(*117*)
		Copper treatment[e] (15 to 100%)	(*25*)
Peroxidase	Sulfite–H_2O_2	ΔpH (0 to 100%)	(*69*)

[a] One-electron oxidation and reduction of substrate are measured.
[b] This means that the one-electron flux increases from 5 to 50% with the increase of $[O_2]$.
[c] Milk xanthine oxidase can be prepared as a xanthine dehydrogenase type (*118*).
[d] The one-electron flux does not depend on [Q] in this case.
[e] Sulfhydryl groups of the enzyme are oxidized without changing the NADH-Q reductase activity.

nones are used as acceptor.

$$NAD(P)H + 2Q \rightarrow NAD(P)^+ + 2Q^{-}\cdot + H^+ \quad (10)$$

The free energy is fairly conserved in the above reaction as compared with the two-electron transfer reaction:

$$NAD(P)H + Q + H^+ \rightarrow NAD(P)^+ + QH_2 \quad (11)$$

The standard free energy to be saved can be calculated by

$$\Delta E_o' = -1.36 \log K_s \quad (12)$$

where K_s is a free-radical formation constant, represented as

$$K_s = \frac{[Q^{-}\cdot]}{[QH_2][Q]} \quad (13)$$

the redox potential (E_2) for $Q/Q^{-}\cdot$ is

$$E_2 = E_m + (RT/2F) \ln K_s \quad (14)$$

where, E_m is mean redox potential for QH_2/Q (*122*). $Q^{-}\cdot$ is a strong reductant and can reduce cytochromes and molecular oxygen at considerably high rates. Therefore, if quinones in respiratory chains are exposed to molecular oxygen, electrons will leak out to the oxygen to form superoxide radicals.

The quinone *in vivo* is a component of respiratory chains and embeded in membranes. It is reasonable to assume that the quinone is reduced to the semiquinone in membrane as well as in solution. In mitochondria, ubiquinone may act as translocator of protons (*123–125*). There may be striking difference in the kinetics of electron transfer from flavin to quinone between in solution and in membrane. In solution, the semiquinone formed in the first one-electron transfer from flavin is freed from the enzyme and the second electron of the flavoprotein is transfered to a fresh quinone molecule (*41,42*). In contrast, it is possible that the same quinone molecule accepts two electrons successively from the enzyme. The reduction of quinone in respiratory chains can be formulated as shown in Eq. (15):

$$Q \xrightarrow[H^+]{H(\text{flavoprotein})} Q^{-}\cdot \xrightarrow[H^+]{H(\text{flavoprotein})} QH_2 \quad (15)$$

As the quinone is mobile in membrane, it will be possible that the first and the second reduction of quinone at opposite sites of membrane, causes proton translocation. The translocation of two protons per electron may also be possible as shown in Eq. (16):

$$Q^{-}\cdot \xrightarrow[H^+]{H(\text{flavoprotein})} QH_2 \xrightarrow[2H^+]{-e(\text{cytochromes})} Q^{-}\cdot \quad (16)$$

if the semiquinone-hydroquinone cycle shuttles between the opposite site of membrane.

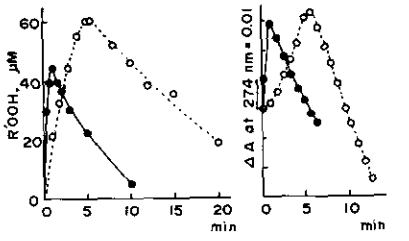

FIGURE 7. Formation and decay of hydroperoxide (R'OOH) derived from indole-3-acetate. The amount of R'OOH can be measured either from its reaction with peroxidase (left) or from the increase of absorbance at 274 nm (right). The solid and dotted lines show time courses for horseradish peroxidases C and A, respectively (*136*).

Metabolism

Hydroperoxides are found to be intermediates in metabolic paths. The peroxidase is a typical enzyme to be involved in the metabolism of hydroperoxides. Therefore, it is of interest to look at the role of peroxidase reactions involved in hydroperoxide metabolism

Hydroperoxide Derived from Indole-3-acetic Acid (IAA). The mechanism of oxidation of a plant hormone, IAA has been a subject of investigation by many plant physiologists (*126–135*). It is now accepted that free radical species which are formed through catalysis of peroxidase are intermediates in the degradation of IAA (*128–134*). The stoichiometry of the peroxidase-catalyzed formation of free radicals has been confirmed by using ferric *o*-phenanthroline complex as an electron acceptor for the free radical (*40*).

$$2\ IAA + H_2O_2 \xrightarrow{\text{Peroxidase}} 2\ IAA\cdot + 2H_2O \quad (17)$$

$$IAA\cdot + \text{ferric complex} \rightarrow \text{dehydro IAA} + \text{ferrous complex} \quad (18)$$

It is also found (*136*) that a hydroperoxide derived from IAA is a good substrate for peroxidase. There is, more or less, a lag in the oxidase reaction and the oxidase reaction in the steady state can be formulated as shown in Eqs. (19)–(24) (*136*):

$$3 + R'OOH \rightarrow 5 + R'OH \quad (19)$$

$$5 + RH \rightarrow 4 + R\cdot \quad (20)$$

$$4 + RH \rightarrow 3 + R\cdot \quad (21)$$

$$R\cdot + O_2 \rightarrow R'OO\cdot + CO_2 \quad (22)$$

$$R'OO\cdot + RH \rightarrow R'OOH + R\cdot \quad (23)$$

$$R'OOH \rightarrow X \rightarrow \text{3-methyleneoxindole} \quad (24)$$

where RH and R'OH are IAA and indole-3-methanol, respectively. The hydroperoxide (R'OOH) accumulates during the reaction (Fig. 7) and reacts with 3 very fast. Therefore, the formation of 5 becomes no more rate-limiting shortly after the reaction starts. Then, like ordinary peroxidase reactions, the reduction of 4 by RH is rate-limiting, and most of the peroxidase exists at 4

during the reaction (*136*).

It may be emphasized from the physiological point of view that in the presence of oxygen the peroxidase-IAA system yields strong oxidizing species. Since it has been reported that the formation of ethylene (plant hormone) is promoted by the addition of IAA (*137–141*), there is a possibility that the ethylene formation is promoted by such oxidizing species.

It should be noted also that peroxidases catalyze oxygenation of various compounds in plant (*142–144*). The reaction may be accelerated in the presence of IAA.

Prostaglandin Synthesis. Prostaglandins are produced from arachidonic acid by a cascade of enzymes. The first step is the conversion of arachidonic acid to prostaglandin G (a hydroperoxide form) by cyclooxygenase. The spin-trapping method has shown the formation of a carbon centered free radical of arachidonic acid in the reaction by ram seminal vesicle microsomes (*145*). The next step is the conversion from prostaglandin G to H catalyzed by a peroxidaselike enzyme. However, a purified enzyme preparation that catalyzes the above two reactions has not been resolved yet (*146,147*) and is termed prostaglandin endoperoxide synthetase. The formation of free radicals has been demonstrated during the peroxidatic oxidation of various electron donors by the enzyme (*35–37*).

The enzyme requires heme for the activities and the mechanism of the reactions can be compared with that of IAA oxidase reaction catalyzed by horseradish peroxidase. On the basis of the assumption that the two reactions are essentially of the same type, it will be suggested for the prostaglandin synthesis that the reactions of cyclooxygenase and peroxidase are catalyzed by the same enzyme; that the peroxidase reaction causes dehydrogenation of arachidonic acid to form its free radical; that the free radical reacts with molecular oxygen to form prostaglandin G via a chain reaction, and that a trace amount of hydroperoxide initiates the peroxidase reaction. This mechanism is the same as that proposed by O'Brien and Rahimtula (*148*).

Although the mechanism of prostaglandin endoperoxide synthetase may not be explained by simple chain reactions involving the hydroperoxy radical, it is of interest to note that a somewhat similar problem has long been discussed also in the mechanism of IAA oxidase reaction.

Thyroxine Synthesis. Thyroid peroxidase catalyzes iodination of tyrosine and oxidative coupling of two diiodotyrosines. It is confirmed that the oxidation of iodide occurs in a two-electron process and that of diiodotyrosine occurs by way of one-electron transfer.

$$3 + H_2O_2 \rightarrow 5 \quad (25)$$

$$5 + I^- \rightarrow 3{\cdot}I^+ + H_2O \quad (26)$$

$$\text{Tyr} + 3{\cdot}I^+ \rightarrow \text{monoiodo Tyr} + 3 \quad (27)$$

$$\text{Monoiodo Tyr} + 3{\cdot}I^+ \rightarrow \text{diiodo Tyr} + 3 \quad (28)$$

$$2(\text{Diiodo Tyr}) + 5 \rightarrow 2(\text{diiodo Tyr}{\cdot}) + 3 \quad (29)$$

$$2(\text{Diiodo Tyr}{\cdot}) \xrightarrow{I^-} \text{thyroxine} \quad (30)$$

The two-electron oxidation of halide ions has been confirmed with horseradish peroxidase (*70,71*), lactoperoxidase (*149*) and thyroid peroxidase (*149,150*). It is in general believed that peroxidases catalyze one-electron oxidation of phenolic compounds. We have recently found a curious fact that thyroid peroxidase catalyzes one-electron oxidation of diiodotyrosine and two-electron oxidations of monoiodotyrosine and tyrosine (*149,151*). Horseradish peroxidase and lactoperoxidase both catalyze one-electron oxidations of the above three molecules (*151*). The ability of thyroid peroxidase of distinguishing the one- and two-electron processes is so peculiar that biochemists will tend to consider its physiological meaning. We have concluded (*150*) that the switch-over of the mechanism from the two-electron to the one-electron type with the increase of the iodide content in tyrosine derivatives is a kind of regulation so that the enzyme synthesizes preferentially thyroxine at the expense of a limited amount of substrates. Thyroxine must be synthesized, *in vivo*, in the presence of limited amounts of hydrogen peroxide, iodide or tyrosine residues of thyroglobulin. For economy, thyroxine should be formed without accumulation of monoiodotyrosine and diiodotyrosine.

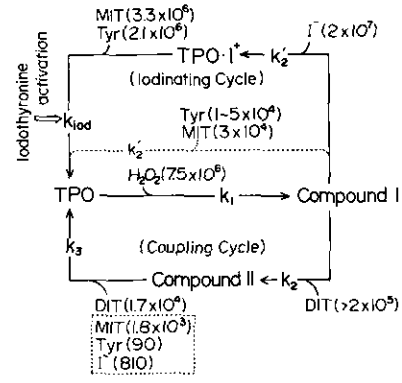

FIGURE 8. One-electron (bottom) and two-electron (upper) oxidation pathways in thyroxine synthesis by thyroid peroxidase. Numerals in parentheses denote second-order rate constants. The mechanism is converted from the upper to the bottom cycle as diiodotyrosine (DIT) accumulates. Reactions shown by dotted lines are not involved in the main path of thyroxine synthesis (*150*).

As shown in Figure 8, tyrosine and monoiodotyrosine compete with iodide but inhibition of iodination by tyrosine and monoiodotyrosine is not serious because of high rate of reaction of iodide with 5. As diiodotyrosine accumulates, the reaction of 5 with diiodotyrosine cannot be neglected, and, in this case, the enzyme is converted into 4, which reacts with iodide very slowly. The values of rate constant tell us that as the concentration of diiodotyrosine increases the iodination is inhibited and the coupling of diiodotyrosine is accelerated.

Miscellaneous

The redox property of the ascorbate system is still mysterious in many points, in spite of numerous experimental data. The difficulty in studying this redox

property is partly due to the instability of dehydroascorbate. The redox property of semidehydroascorbate appears to differ from that of semiquinones. E_o' for the dehydroascorbate/semidehydroascorbate couple is calculated from Eq. (14) to be −200 mV (*152*). Semiquinones having this E_o' value can reduce molecular oxygen (*153,153*), but semidehydroascorbate cannot. Dismutation of semidehydroascorbate is very fast, but the reduction of cytochrome c by semidehydroascorbate is relatively slow (*155*). There is a widely distributed enzyme system that reduces semidehydroascorbate to ascorbate at the expense of NADH (*6–13,156*). The bicyclic structure of semidehydroascorbate (*157,158*) might be related to its abnormal reactivity and the characteristic feature of ascorbate redox system.

The generation of superoxide anions during phagocytosis and action of antitumor quinones is an interesting subject which is dealt with in other chapters. Some peculiar enzyme catalyses in which the free-radical mechanism is involved have been recently reported (*159–161*). A tyrosine radical is involved in the ribonucleotide reductase reaction (*159,160*) and pyrroloquinoline semiquinone is involved in the methanol dehydrogenase reaction (*161*). Since quinoproteins are widely distributed in bacteria (*162*), the free radical mechanism will actually be more popular in enzyme catalyses.

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